Allergology International (1997) **46**: 255–260

Original Article

Inhibitory action of azelastine hydrochloride on the induction of mast cells from normal mouse splenocytes

Kazuhito Asano,[1] Masashi Taki,[2] Sumitaka Matsuo[3] and Kaneo Yamada[2]

[1]Department of Medical Biology, School of Medicine, Showa University, Tokyo, [2]Department of Pediatrics, School of Medicine, St Marianna University and [3]Department of Clinical Hematology, School of Allied Health Sciences, Kitasato University, Kanagawa, Japan

ABSTRACT

The influence of azelastine (AZ) on murine mast cell induction was examined by *in vitro* cell culture techniques. Splenocytes from normal BALB/c mice suspended in RPMI-1640 medium supplemented with interleukin-3 were cultured in the presence or absence of AZ. The number of mast cells increased gradually with culture time and reached a peak on the 16th day when cells were cultured without AZ. The addition of 1.0 μg/mL AZ to the culture medium resulted in complete suppression of mast cell growth, but a lower concentration of AZ (0.5 μg/mL) had no demonstrable effects on the growth of mast cells. Azelastine induced apoptotic cell death in mast cells when cells were cultured in the presence of 1.0 μg/mL AZ for more than 24 h. However, this apoptosis-inducing activity of AZ was not observed in either eosinophils or neutrophils that were obtained from mouse peritoneal cavity.

Key words: apoptosis, azelastine, *in vitro* mast cell growth.

INTRODUCTION

Mast cells are prominent in a variety of inflammatory reactions and have been shown to be one of the principal cellular components of allergic reactions. The allergic reactions occur when antigen-specific IgE bound to the high affinity IgE receptor (Fcε RI) on the surface of mast cells is aggregated by binding to a multivalent antigen.[1] The cross-linking of cell-bound IgE, in turn, aggregates the Fcε RI that activates the mast cell.[1] Activation of mast cells leads to three different reactions: (i) exocytosis of granules containing histamine and other chemical mediators;[2] (ii) synthesis and secretion of inflammatory cytokines;[3] and (iii) synthesis and secretion of newly formed mediators, such as prostaglandins and leukotrienes.[1] Based on these observations, many types of drugs have been developed to prevent the membrane change of mast cells associated with the secretion of mediators and these drugs are used therapeutically. However, mast cells are well known to be derived from pluripotent progenitor cells that can be found in highest numbers in the bone marrow and then reside in the blood, peripheral mucosa and peripheral tissues. It is also recognized that inflammatory responses in these sites bring about differentiation and proliferation of the progenitor cells and accumulation of mature mast cells. However, there are few reports about whether or not anti-allergic drugs used in therapy inhibit mast cell differentiation.[4,5]

Azelastine hydrochloride (AZ), a phthalazinone derivative, is widely used for the management and treatment of asthma and atopic allergy. The mechanisms of action of AZ have been extensively examined and it has been reported that AZ exerts inhibitory effects on allergic and non-allergic release of chemical mediators, such as histamine, serotonin and platelet-activating factor (PAF) from mast cells, eosinophils and other cells.[6–8] Azelastine has also been shown to act as a functional antagonist of PAF[9] and leukotrienes.[6] However, no information about the effects of AZ on mast cell proliferation and differentiation is yet available. Therefore, we designed the present study to evaluate the *in vitro* effects of AZ on mast cell

Correspondence: Dr K Asano, Department of Medical Biology, School of Medicine, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142, Japan.

Received 2 October 1996. Accepted for publication 2 June 1997.

growth using cell culture techniques and found that AZ strongly inhibits the differentiation of mast cells from normal mouse splenocytes.

METHODS

Mice

Male BALB/c mice (5 weeks of age) were purchased from Charles River Japan Inc. (Atsugi, Japan).

Preparation of conditioned medium

To prepare the conditioned medium containing interleukin (IL)-3, WEHI-3 cells donated from Riken Gene Bank (Tsukuba, Japan) were cultured in RPMI-1640 medium (GIBCO BRL, Life Technologies Inc., Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS; Flow Laboratories, North Ryde, NSW, Australia), 5×10^{-5} mol/L 2-mercaptoethanol (2ME), 25 mmol/L HEPES, 10 mmol/L L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (RPMI) at a concentration of 1×10^5 cells/mL at 37°C in a humidified atmosphere with 5% CO_2 in air. Cell-free culture supernatants were obtained 72 h after culture and were used as a conditioned medium.[10]

Agent

Azelastine (AZEPTIN®) was kindly supplied by EISAI Co. Ltd (Tokyo, Japan) as a preservative-free pure powder. The agent was dissolved in boiling distilled water at a concentration of 1.0 mg/mL. After cooling, the solution was diluted with RPMI containing 50% conditioned medium (complete medium) at suitable concentrations described below.

Cell preparation and cell culture

The spleen cell suspension was prepared as described previously.[5,11] Cells were adjusted to 1×10^7 cells/mL in complete medium. The cells (1.0 mL) were introduced into each well of a 24-well cluster plate (Nunc, Kamstrup, Denmark) in duplicate and were incubated at 37°C in a humidified atmosphere with 5% CO_2 in a total volume of 2.0 mL. Half the culture medium was changed every 4–5 days.[5,11]

Preparation of peritoneal leukocytes

Peritoneal exudate cells were collected from mice on day 21 after intraperitoneal injection with 50 μL packed tetrathyridia of *Mesocestoides corti*.[12,13] The cell suspension was placed in plastic petri dishes (IWAKI GLASS Co. Ltd, Chiba, Japan) and were incubated for 2 h at 37°C in a humidified atmosphere with 5% CO_2. Non-adherent cells were collected, suspended in RPMI supplemented with 200 U/mL recombinant mouse IL-5 (Genzyme Corp., Boston, MA, USA) and were used as eosinophil-enriched cell populations. Eosinophils were counted in a hemocytometer with Hinkelman's solution (Muto Pure Chemicals Co., Tokyo, Japan) and cell viability was checked by trypan blue dye exclusion. The purity of eosinophils used was > 96% with viability above 98.0%. Neutrophils were harvested 5 h after intraperitoneal injection of 2.0 mL of 0.2% casein (Wako Pure Chemical Co. Ltd, Osaka, Japan).[13] The purity of neutrophils used was > 95% as judged by morphological examination after staining with Giemsa solution (Wako Pure Chemical Co. Ltd). The viability of the cells was greater than 98%. Cells were suspended in RPMI supplemented with 200 U/mL recombinant mouse granulocyte macrophage-colony stimulating factor (Genzyme Corp.) and were used as neutrophil-enriched cell populations.

Treatment of cells with AZ

Azelastine was added to cells at the seeding stage and then every 4–5 days, when the culture medium was replaced with fresh medium. To examine the effects of AZ at different stages in cultured cells, another series of experiments was performed, in which the effects of the addition of AZ to cell cultures at the seeding stage and at 14 days after seeding were compared.

Cell counts

Every 4–5 days, the growth of cells was monitored by counting cells in a hemocytometer. Cell viability was checked by the trypan blue dye exclusion test. Mast cells were counted according to the method of Gilbert and Ornstein.[14] Results are expressed as the mean±SD of three different experiments.

Mast cell separation

Cultured mast cells were separated from dead cells and small lymphocytes by Histopaque-1077 (Sigma Chemical Co., St Louis, MO, USA) density gradient centrifugation. Cultured cells were collected and washed twice with FCS-free RPMI-1640 medium (GIBCO BRL) at 800 *g* for 10 min at 25°C. Cells were suspended in the

medium, layered on Histopaque-1077 and centrifuged at 1200 *g* for 30 min at 25°C. Separated cells were then collected and resuspended in the medium. More than 98% of separated cells were stained following the method of Gilbert and Ornstein.[14]

DNA fragmentation assay

DNA fragmentation was assayed by agarose gel electrophoresis.[15–17] Azelastine-treated and -non-treated cells (5×10^5 cells/sample) were lysed by 20 μL lysis buffer containing 50 mmol/L Tris, 10 mmol/L EDTA and 0.5% (w/v) sodium *N*-lauroyl sarcosinate. Solutions were digested with 0.5 mg/mL RNase A (Amersham International plc, Buckinghamshire, UK) for 60 min at 50°C, followed by 0.5 mg/mL proteinase K (Amersham) for 60 min at 50°C. The resulting DNA preparations were directly applied to 1.8% agarose gel electrophoresis in Tris-borate EDTA (TBE) buffer (89 mmol/L Tri, 89 mmol/L boric acid, 2 mmol/L EDTA, pH 8.1). Separation of fragments was performed at 100 V for 1.0 h. Ethidium bromide was used to visualize the DNA and photographs of the gel were taken under UV irradiation. λDNA Eco T14I digest (Takara Co. Ltd, Kyoto, Japan) was used as a DNA marker to estimate the size of DNA fragments.

Statistical analysis

The significance of differences between control and experimental groups was determined by the Student's *t*-test.

RESULTS

Influence of AZ on *in vitro* mast cell growth

To examine the effect of AZ on mast cell growth, 1×10^7 splenocytes were cultured in the presence or absence of AZ and the numbers of viable cells and mast cells were counted. Experiments were repeated twice and results are expressed as the mean±SD of three different experiments. The number of viable cells gradually decreased with culture time (Fig. 1a). It is also apparent from Fig. 1a that cell viability was reduced dramatically when much higher doses of AZ (5.0 and 10.0 μg/mL) were added to the culture medium due to the cytotoxicity of the agent for long-term cell culture. When splenocytes were cultured without AZ, the mast cell number increased gradually, peaked on the 16th day and declined thereafter (Fig. 1b). A statistically significant ($P < 0.01$) and dose-dependent inhibition of mast cell growth was observed when splenocytes were exposed throughout the

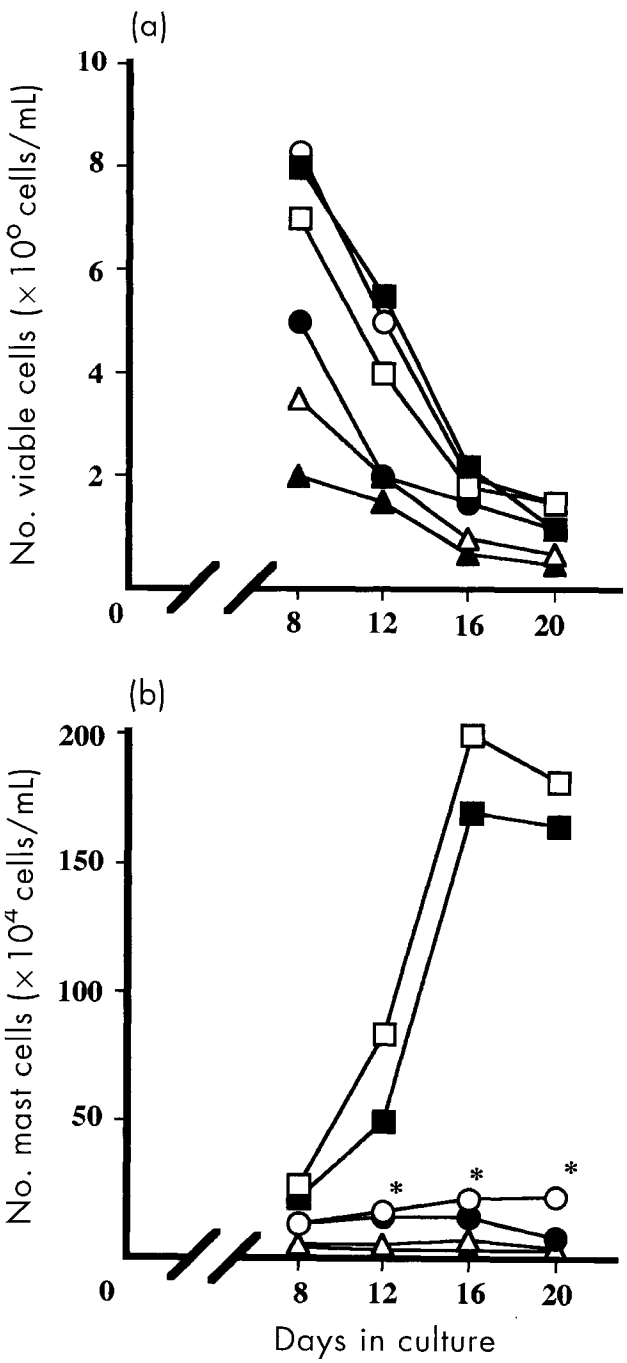

Fig. 1 Effect of azelastine on (a) viable cells and (b) mast cell induction in cultured murine splenocytes. Azelastine ((■), 0.5 μg/mL; (○), 1.0 μg/mL; (●), 2.0 μg/mL; (△),5.0 μg/mL; (▲), 10.0 μg/mL) was added to cell cultures at the time of seeding 1×10^7 cells and then every 4–5 days, when the culture medium was replaced with fresh medium. Each point represents a mean of three different experiments; bars indicating SD have been omitted for clarity. (□), non-treated control. *P <0.01 compared with non-treated control.

entire culture period to AZ: as the concentration of the agent in the culture medium was increased, the number of mast cells decreased (Fig. 1b).

The second set of experiments was designed to examine the effects of AZ at different stages of mast cell growth. To do this, 0.5 and 1.0 μg/mL AZ was added to the cultures at seeding of splenocytes or 14 days after seeding and the mast cell numbers were counted on the 20th day. As shown in Fig. 2, the addition of AZ at seeding significantly inhibited mast cell growth. Although the inhibition of mast cell growth was not observed when 0.5 μg/mL AZ was added to the culture on the 14th day, a higher dose of AZ (1.0 μg/mL) significantly inhibited mast cell growth.

Induction of DNA fragmentation in cultured-mast cells

The third set of experiments was undertaken to examine whether AZ produced apoptotic mast cell death and resulted in the inhibition of mast cell growth. Mast cells collected on the 16th day were cultured in the presence or absence of AZ (1.0 μg/mL) for various periods. The DNA was extracted and electrophoresed. The DNA extracted from mast cells cultured in the absence of AZ (control) showed no detectable fragmentation (Fig. 3). In contrast, when mast cells were cultured with AZ for 24 or 48 h (but not 6 and 12 h), the chromatin DNA showed characteristic 'ladders', which are features of inter-nucleosomal degradation of DNA (Fig. 3). These results raise the question whether AZ can also induce apoptotic cell death in both eosinophils and neutrophils, which are well known to be final effector cells in allergic responses. Therefore, the final set of experiments was designed to examine the ability of AZ to induce apoptotic cell death in eosinophils and neutrophils. An apoptosis-inducing activity of AZ was not observed in these cells, even when the cells were cultured in the presence of 1.0 μg/mL AZ for 48 h (Fig. 3).

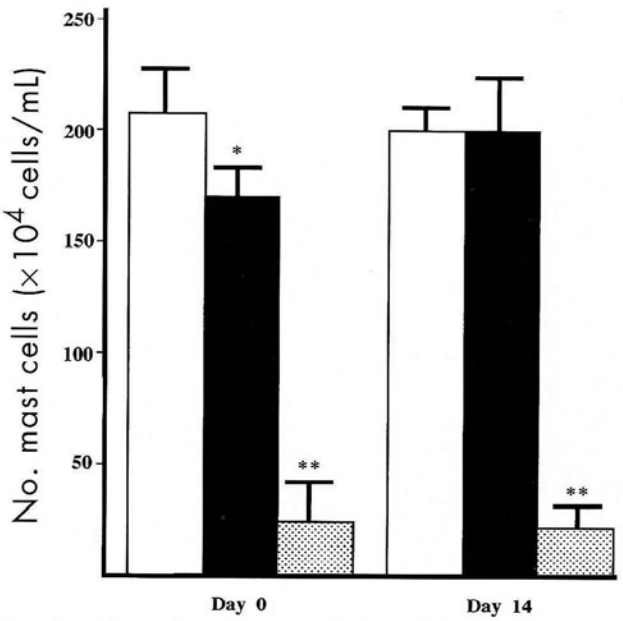

Fig. 2 Effect of azelastine added at different times after seeding 1 × 10^7 splenocytes. Azelastine was added to the culture medium on days 0 and 14 and the number of mast cells was counted on day 20. Each point represents the mean±SD of three different experiments. (□), non-treated control; (■), cells treated with 0.5 μg/mL azelastine; (⊡), cells treated with 1.0 μg/mL azelastine. *$P < 0.05$, **$P < 0.01$, compared with non-treated control.

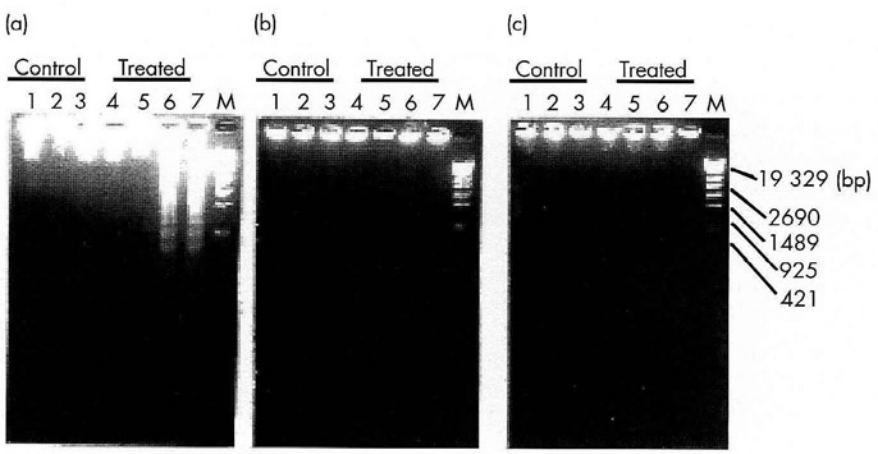

Fig. 3 Effect of azelastine on DNA fragmentation in (a) mast cells, (b) eosinophils and (c) neutrophils. Splenocytes (1 × 10^7 cells) were cultured for 16 days and mast cells were induced. Eosinophils and neutrophils were obtained from the mouse peritoneal cavity. These cells (1 × 10^6 cells/mL) were then cultured in the presence (treated) or absence (control) of 1.0 μg/mL azelastine for various periods. The DNA was extracted from 5 × 10^5 cells and was assayed for DNA fragmentation in 1.8% agarose gel electrophoresis. Lanes 1–3, non-treated cells cultured for 12, 24 and 48 h, respectively; lanes 4–7, treated cells cultured for 6, 12, 24 and 48 h, respectively; M, DNA molecular weight standards.

Discussion

Mast cells are located in areas where they are most likely to encounter allergens, such as submucosally in the respiratory tracts. Following the induction of allergic diseases, mast cells are activated by allergen cross-linking of Fcε RI.[1] Subsequently, a number of chemical mediators are released.[1–3] These mediators promote the immediate onset of bronchoconstriction, mucus secretion and production of new mediators, such as PAF and leukotrienes.[6–9] It is well accepted that allergic patients have elevated mast cell (basophil) levels in their circulation and in mucosal tissues compared with normal subjects.[18,19] Recently, we reported that IPD-1151T, a newly synthesized anti-allergic drug, could inhibit mast cell growth from normal mouse splenocytes when cells were cultured *in vitro* in the presence of the agent.[4,5] The lack of any inhibitory action of sodium chromoglycate, one of the best-known anti-allergic drugs, on mast cell growth has also been reported.[5] Although many kinds of anti-allergic drugs are now used clinically, there are as yet limited data available on the effects of these agents on mast cell growth. Therefore, we chose AZ, a widely used (particularly in Japan) anti-allergic drug, and examined whether this agent also had inhibitory effects on mast cell growth.

The present results show that AZ inhibits *in vitro* mast cell growth from normal mouse splenocytes when splenocytes are exposed throughout the entire culture period to AZ. This inhibitory action was also observed when AZ was added to cultures 14 days after seeding of splenocytes. Our previous work has shown that when mouse splenocytes are cultured with IL-3, mucosal-type immature mast cells were detected on day 8 of culture. These immature mast cells then gradually differentiated and almost all cells reached maturity 10–12 days after seeding.[5,11] Together with these reports, the results presented in Figs 1 and 2 may be interpreted to indicate that AZ exerts its inhibitory effects on both immature (including mast cell progenitors) and mature mast cells and results in suppression of mast cell growth.

Glucocorticoid (steroid) treatment has been shown to lead to a reduction of the number of granulocytes, including mast cells and eosinophils, in allergic patients,[20–22] due to, in part, the ability of glucocorticoids to induce apoptotic cell death in granulocytes.[23] Therefore, we examined whether AZ induced apoptotic cell death in cultured mast cells and resulted in the inhibition of mast cell growth. The results shown in Fig. 3 clearly show that AZ induces nucleosome-sized DNA fragmentation in mast cells when cells are cultured in the presence of 1.0 mg/mL AZ for 24–48 h. In contrast, eosinophils and neotrophils showed no intranucleosomal DNA cleavage following treatment with AZ. The reason for this discrepancy is not clear at present. Apoptosis is generally thought to involve the early activation of endonucleases that preferentially cleave DNA in the linker regions between nucleosomes, resulting in the production of fragments that are multiples of a unit comprising 180–200 bp.[15–17] It is also reported that there is a variety of endonucleases (e.g. DNase I and DNase II etc.) whose characteristics, including activation mechanisms, depend on cell types.[16] Therefore, the results shown in Fig. 3 may indicate that the type of endonuclease(s) in mast cells is different to that in both neutrophils and eosinophils and that endonuclease activity in cells examined in the present study is regulated by different mechanisms. A more detailed study to clarify the mechanisms by which AZ induces apoptotic mast cell death is underway in our laboratory. The present results clearly indicate that AZ inhibits *in vitro* mast cell growth from normal mice spleen cells. However, before drawing the conclusion that the attenuating effect of AZ on allergic syndromes may be explained, in part, by the inhibitory action of AZ on mast cell growth *in vitro*, it is necessary to examine whether orally administered AZ is able to inhibit mast cell growth *in vivo*. Because it has been reported that the plasma concentration of AZ in asthmatic patients was approximately 2 ng/mL when they were treated with the agent at a dose of 4 mg that is a therapeutic dose of AZ to produce beneficial responses in the patients.[24]

The properties of mouse mast cells induced *in vitro* by IL-3 are well known to be different to those of human mast cells; for example, the former cells express IL-3 receptors on the cell surface and respond well to IL-3 stimulation, while the latter cells lack the responsiveness to IL-3 stimulation because of the absence of the appropriate receptors.[25] It has also been reported that chemical mediators contained in murine mast cells grown *in vitro* are different to those in human mast cells.[26–28] Therefore, it also remains to be examined whether the suppressive effects of AZ on mouse mast cell growth are also observed in human mast cell (including basophil) growth.

Acknowledgements

We thank Dr T Ohno (Cell Bank, Riken Gene Bank, Ibaragi, Japan) for providing the WEHI-3 cells. This study was supported in part by EISAI Co. Ltd (Tokyo, Japan).

REFERENCES

1 Bonham CA, Thomson AW. Anti-inflammatory agents in allergic diseases. *Clin. Exp. Immunol.* 1995; **102**: 1–5.
2 Lagnoff D. Contribution of electron microscopy to the study of mast cells. *J. Invest. Dermatol.* 1972; **58**: 296–311.
3 Bradding P. Human mast cell cytokines. *Clin. Exp. Allergy* 1996; **26**: 13–19.
4 Konno S, Adachi M, Asano, K. *et al.* Inhibitory effects of IPD-1151T (Suplatast-tosilate) on mast cell induction from normal mouse spleen cells. *Jpn. J. Allergol.* 1992; **41**: 1488–91.
5 Konno S, Adachi M, Asano K *et al.* Suppressive effect of IPD-1151T (Suplatast-tosilate) on induction of mast cells from normal mouse splenocytes. *Eur. J. Pharmacol.* 1994; **259**: 15–20.
6 Katayama S, Akimoto N, Shinoya H, Morimoto T, Katoh Y. Anti-allergic effect of azelastine hydrochloride on immediate type hypersensitivity reactions *in vivo* and *in vitro*. *Arzneimittelforschung* 1981; **31**: 1196–203.
7 Chand N, Nolan K, Diamantis W, Perhach JL, Sofia RD. Inhibition of leukotrien (SRS-A)-mediated acute lung anaphylaxis by azelastine in guinea pigs. *Allergy* 1986; **41**: 473–8.
8 Little MM, Casale TB. Azelastine inhibits both human lung and basophil degranulation. *J. Allergy Clin. Immunol.* 1987; **79**: 204A.
9 Achterrath-Tuckermann U, Weisher CH, Szelenyi I. Azelastine, a new antiallergic/antiasthmatic agent, inhibits PAF-acether-induced platelet aggregation, paw edema and bronchoconstriction. *Pharmacology* 1988; **36**: 265–71.
10 Ralph P, Nokoiz I. Direct toxic effects of immunopotentiators on monocytic, myelomonocytic, and histocytic or macrophage tumor cells in culture. *Cancer Res.* 1977; **37**: 546–50.
11 Konno S, Adachi M, Asano K, Okamoto K, Takahashi T. Inhibitory effect of interferon-beta on mouse spleen-derived mast cells. *Mediators Infla.* 1993; **2**: 243–6.
12 Owhashi M, Ishii A. Purification and characterization of a high molecular weight eosinophil chemotactic factor from *Schistosoma japonicum* eggs. *J. Immunol.* 1982; **129**: 2226–31.
13 Niwa A, Miyazato T. Reactive oxigen intermediates from eosinophils in mice infected with *Hymenolepis nana*. *Parasite Immunol.* 1996; **18**: 285–95.
14 Gilbert H, Ornstein L. Basophil counting with a new staining method using alcian blue. *Blood* 1975; **59**: 284–92.
15 Shiota-Y F, Sakagami H, Kuribayashi N *et al.* Endonuclease activity and induction of DNA fragmentation in human myelogenous leukemic cell lines. *Anticancer Res.* 1995; **15**: 259–66.
16 Sakagami H, Kuribayashi N, Iida M *et al.* Induction of DNA fragmentation by tannin- and lignin-related substances. *Anticancer Res.* 1995; **15**: 2121–8.
17 Sakagami H, Kuribayashi N, Iida M *et al.* The requirement for and mobilization of calcium during induction by sodium ascorbate and by hydrogen peroxide of cell death. *Life Sci.* 1996; **58**: 1131–8.
18 Otsuka H, Denburg JA, Dolovichi J *et al.* Heterogeneity of metachromatic cells in human nose: Significance of mucosal mast cells. *J. Allergy Clin. Immunol.* 1985; **76**: 695–702.
19 Gibson PG, Dolovichi J, Girgis-Gabardo A *et al.* The inflammatory response in asthma exacerbation: Changes in circulation eosinophils, basophils and their progenitors. *Clin. Exp. Allergy* 1990; **20**: 661–8.
20 Spector SL, English G, Jones L. Clinical and nasal biopsy response to treatment of perennial rhinitis. *J. Allergy Clin. Immunol.* 1980; **66**: 129–37.
21 Gomez E, Clague JE, Gatland D, Davies RJ. Effect of topical corticosteroids on seasonally induced increases in nasal mast cells. *BMJ* 1988; **296**: 1572–3.
22 Turner CR, Spannhake EW. Acute topical steroid administration blocks mast cell increase and the late asthmatic response of the canine peripheral airways. *Am. Rev. Respir. Dis.* 1990; **141**: 421–7.
23 Schleimer RP. The effects of anti-inflammatory steroids on mast cells. In: Kaliner MA, Metcalf DD (eds). *The mast cell in health and diseases*. New York: Marcel Dekker Inc., 1993; 483–511.
24 Spector SL, Perhach JL, Rohr AS *et al.* Pharmacodynamic evaluation of azelastine in subjects with asthma. *J. Allergy Clin. Immunol.* 1987; **80**: 75–80.
25 Valent P, Besemer J, Sillaber CH *et al.* Failure to detect IL-3-binding sites on human mast cells. *J. Immunol.* 1990; **145**: 3432–7.
26 Reynolds DS, Stevens RL, Lane WS *et al.* Different mouse mast cells populations express various combinations of at least six distinct mast cell serine proteases. *Proc. Natl Acad. Sci. USA* 1990; **87**: 3230–4.
27 McNeil HP, Reynolds DS, Schiller V *et al.* Isolation, characterization, and transcription of the gene encoding mouse mast cell protease. *Proc. Natl Acad. Sci. USA* 1992; **89**: 11 174–8.
28 Schwartz LB. Mast cell: Function and contents. *Curr. Opin. Immunol.* 1994; **6**: 91–7.